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(54) Title: EXPRESSION OF RETROVIRUS GAG PROTEIN IN EUKARYOTIC CELLS

(57) Abstract

This invention is a recombinant DNA molecule for expression of HIV-1 gag precursor protein in eukaryotic cells which comprises the HIV-1 gag coding region and regulatory regions which allow for expression of the gag DNA when said DNA is used to transform eukaryotic host cells. Also described are methods for producing HIV-1 gag protein, method for producing a recombinant DNA molecule, and the HIV-1 gag protein product produced by host cells transformed with the recombinant DNA described in the instant application.

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Expression of Retrovirus gag Protein
in Eukaryotic Cells

Field of the Invention

10 This invention relates to expression of proteins in eukaryotic cells. More particularly it relates to the expression of immunodeficiency virus gag precursor protein.

Background of the Invention

15 Retroviruses, that is, viruses within the family, Retroviridae, are a large family of enveloped, icosohedral viruses of about 150 nm having a coiled nucleocapsid within the core structure and having RNA as the genetic material. The family comprises the oncoviruses such as the
20 sarcoma and leukemia viruses, the immunodeficiency viruses and the lentiviruses.

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1 Human Immunodeficiency Virus (HIV), the etiologic agent of
acquired immune deficiency syndrome (AIDS) and related
disorders, is a member of the Retroviridae family. There exist
several isolates of HIV including human T-lymphotropic virus
5 type-III (HTLV-III), the lymphadenopathy virus (LAV) and the
AIDS-associated retrovirus (ARV) which have been grouped in
type 1. Related immunodeficiency viruses, include HIV type 2,
which was shown recently to be associated with AIDS in West
Africa. Other immunodeficiency viruses include the SIV viruses
10 such as SIV_{mac} -BK28.

Molecular characterization of the HIV genome has
demonstrated that the virus exhibits the same overall
gag-pol-env organization as other retroviruses. In addition,
it contains at least five genes that are not found in more
15 ordinary retroviruses: sor, tat3, art/trs, 3'orf and R. The
gag region encodes 3 core proteins, p17, p24 and p16, which are
prepared by cleavage of a 55 kilodalton gag precursor protein
by the HIV protease. The protease is encoded by the pol region.

Recent reports have shown that antibodies to the HIV gag
20 proteins, p17, p24 and p16, are present in human sera from
infected individuals in the United States and Europe and that
antibodies arise early after infection. The presence of these
antibodies declines as the individual proceeds towards AIDS.

The gag protein p17 with its submembrane localization is
25 well positioned to be in close contact with the transmembrane
protein gp41 and the viral membrane and with gag p24 and
possibly gag p15 viral RNA thereby playing a central role in
the conformational changes involved in the viral entry and
uncoating process. Furthermore, gag p17 has been found to have
30 a myristylated N-terminus. Myristylation has been implicated
in virion assembly and transport of viral components to the
plasma membrane. Myristylated proteins are generally localized
in the plasma membrane.

35 Madisen et al., Virology 158:248 (1987), report expression
of the HIV gag protein in Spodoptera frugiperda cell using the

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1 AcMNPV Baculovirus using a DNA sequence comprising 5¹
untranslated sequences and sequences from the pol region in
addition to the gag sequence.

5 Cochran, EP-A-228,036, disclose use of a Baculovirus/insect
expression system to express certain proteins. At pages 17a
and 26, AIDS retrovirus core proteins are mentioned among a
list of proteins which may be produced in such system.

10 Cochran et al., EP-A-265,785, disclose expression of HIV
envelope proteins in insect cells using a Baculovirus
expression system.

15 Kramer et al., Science 231:1580 (1986) and Kramer et al.,
EP-A-230,222, report expression of a gag-pol DNA sequence in
yeast and in vivo cleavage of the precursor by the HIV protease
expressed from this region.

20 Valenzuela et al., Nature 298:347 (1982), report synthesis
of the Hepatitis B Virus surface antigen in yeast and its assembly
into particles.

25 Ellis et al., EP-A-251,460, disclose synthesis of the
Hepatitis B Virus core antigen in yeast and its assembly into
particles.

Adams et al., Cell 49:111 (1987), report synthesis of the
p1 protein of Ty-virus-like particles (VLP's) in yeast and
assembly into particles. The outlines conclude that p1 is a
precursor to the major core proteins of Ty-VLP's and, in this
25 respect, is functionally similar to the gag precursor protein
of retroviruses.

Bishop et al., EP-A-260,090, disclose expression of
Hepatitis B Virus antigens using a baculovirus expression
system.

30 Rusche et al., EP-A-272,858, disclose expression of
proteins derived from HIV GP 160 using a baculovirus expression
system.

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1 In spite of major research efforts in the area of AIDS, there continues to be a need for diagnostic reagents which can be used to monitor disease progression and for agents which can prevent primary infection, such as via immunization, and for
5 agents which can prevent or inhibit secondary infection, such as by cell-to-cell transmission or by free virus infection.

Summary of the Invention

In one aspect, this invention is a recombinant DNA molecule
10 for expression of gag precursor protein in eukaryotic cells which comprises a coding sequence there for operatively linked to a regulatory region which functions in the host cell.

In related aspects, this invention is host cells comprising the recombinant DNA molecule and cultures thereof.

15 In further related aspects, the invention is the gag precursor protein produced by the host cells of the invention, including a HIV core-like particle comprising the gag precursor protein.

In yet further related aspects, the invention is a process
20 for producing the recombinant DNA molecule and the host cell of the invention, a process for producing the gag precursor protein and particles of the invention, and related compositions and methods.

25 These and other aspects of the invention are fully described in the disclosure and Examples which follow.

Detailed Description of the Invention

It has now been found that retroviral gag precursor protein can be expressed in recombinant eukaryotic cells and that such expression can result in production of full-length gag precursor protein without use of pol DNA sequences and without use of 5' untranslated sequences from the virus. Exemplary of such cells are cells from lower eukaryotes such as yeast and fungi and animal cells including insect cells such as
30 Drosophila or Lepidoptera cells; mammalian cell lines;
35 mammalian primary cells, and insects and transgenic animals.

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1 It has also been found, unexpectedly, that the gag
precursor protein can form particles which resemble authentic
gag particles formed in infected human cells in size and other
physical properties and in antigenicity. During a natural
5 retrovirus infection cycle, it appears that gag precursor
protein, known in the case of HIV as p55, is formed largely
into particles comprising predominantly full-length gag
protein. These gag particles can be referred to as pre-core
particles or immature core particles. Then, during viral
10 maturation, the precursor is cleaved into the subunit proteins
known in the case of HIV as p17, p24 and p16. These gag
particles, now comprised predominantly of p17, p24 and p16, can
be referred to as core particles or as mature core particles.
Also during viral maturation, apparently during the budding
15 process, the viral membrane is formed around the pre-core or
core particles. As shown in the Examples below, HIV gag
precursor expressed in recombinant Lepidoptera cells using a
Baculovirus expression system are largely aggregated or
packaged in particles which have physical and biological
20 properties and dimensions similar to those of the core of HIV
particles formed naturally in infected human cells. The
particles of the invention comprise predominantly gag precursor
protein (greater than 90% of all protein in the particles is
full length gag precursor) but nevertheless are recognized
25 after brief treatment with Triton X100 by anti-p17 monoclonal
antibodies (MABs), anti-p24 MABs and anti-p16 MABs in addition
to being recognized by anti-gag polyclonal antibodies from sera
of infected patients. The particles, because they are prepared
by recombinant DNA techniques as disclosed herein, lack viral
30 functions required for viral maturation and replication
especially viral RNA and also, preferably, reverse transcriptase
and protease functions.

35 The recombinant eukaryotic cells of the invention are
engineered to express the gag precursor protein by introduction
into the cells of the recombinant DNA molecule of the
invention. The recombinant DNA molecule of the invention

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- 1 comprises a coding region for the gag precursor protein operatively linked to a regulatory element which functions in the selected host cells. As an aspect of this invention, it has been found that other HIV functions are not required for
- 5 expression of the gag precursor protein and for pre-core-like particle formation. DNA sequences coding for other functions, e.g., for amplification functions, selection markers or maintenance functions, can also be comprised within the recombinant DNA molecule of the invention.
- 10 A DNA coding region for gag precursor protein can be prepared from any of the several immunodeficiency virus genomic clones or gag-pol clones reported in the literature. See, for example, Shaw et al., Science 226:1165(1984); Kramer et al., Science 231:1580(1986). Alternatively, an immunodeficiency
- 15 virus genomic clone can be prepared from virus isolated from clinical specimens by standard DNA cloning techniques. See, for example, Gallo et al., U.S. Patent 4,520,113; Montagnier et al., U.S. Patent 4,708,818. Having cloned a fragment of the genome which comprises the gag coding region, a region which
- 20 codes only for the gag precursor can be prepared by restricting the DNA so as to isolate a portion of the DNA coding region and reconstructing the remaining portions through use of synthetic oligonucleotides, such as described in the Examples, below. Alternatively, a larger fragment comprising the gag coding
- 25 region and additional sequences can be cut back through use of exonucleases. In yet another alternative procedure, the entire coding region can be synthesized using standard automated DNA synthesizers by synthesizing fragments of the coding region and ligating these together to form a complete coding region.
- 30 While use of a coding sequence which lacks the naturally occurring 5' and 3' flanking sequences is preferred, fusion of the coding sequence to other immunodeficiency virus sequences, e.g., envelope protein sequences, is not precluded from the preferred embodiments.

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1 An exemplary coding region for the HIV gag precursor protein has the following sequence.

5	1 ATG GGT GCG AGA GCG TCA GTA TTA AGC CCC GGA GAA Met Gly Ala Arg Ala Ser Val Leu Ser Gly Gly Glu	36
	37 TTA GAT CGA TGG GAA AAA ATT CGG TTA AGG CCA GGG Leu Asp Arg Trp Glu Lys Ile Arg Leu Arg Pro Gly	72
	73 GGA AAG AAA AAA TAT AAA TTA AAA CAT ATA GTA TGG Gly Lys Lys Lys Tyr Lys Leu Lys His Ile Val Trp	108
10	109 GCA AGC AGG GAG CTA GAA CGA TTC GCA GTT AAT CCT Ala Ser Arg Glu Leu Glu Arg Phe Ala Val Asn Pro	144
	145 GGC CTG TTA GAA ACA TCA GAA GGC TGT AGA CAA ATA Gly Leu Leu Glu Thr Ser Glu Gly Cys Arg Gln Ile	180
15	181 CTG GGA CAG CTA CAA CCA TCC CTT CAG ACA GGA TCA Leu Gly Gln Leu Gln Pro Ser Leu Gln Thr Gly Ser	216
	217 GAA GAA CTT AGA TCA TTA TAT AAT ACA GTA GCA ACC Glu Glu Leu Arg Ser Leu Tyr Asn Thr Val Ala Thr	252
	253 CTC TAT TGT GTG CAT CAA AGG ATA GAG ATA AAA GAC Leu Tyr Cys Val His Gln Arg Ile Glu Ile Lys Asp	288
20	289 ACC AAG GAA GCT TTA GAC AAG ATA GAG GAA GAG CAA Thr Lys Glu Ala Leu Asp Lys Ile Glu Glu Glu Gln	324
	325 AAC AAA AGT AAG AAA AAA GCA CAG CAA GCA GCA GCT Asn Lys Ser Lys Lys Ala Gln Gln Ala Ala Ala	360
25	361 GAC ACA GGA CAC AGC AGT CAG GTC AGC CAA AAT TAC Asp Thr Gly His Ser Ser Gln Val Ser Gln Asn Tyr	396
	397 CCT ATA GTG CAG AAC ATC CAG GGG CAA ATG GTA CAT Pro Ile Val Gln Asn Ile Gln Gly Gln Met Val His	432
	433 CAG GCC ATA TCA CCT AGA ACT TTA AAT GCA TGG GTA Gln Ala Ile Ser Pro Arg Thr Leu Asn Ala Trp Val	468
30	469 AAA GTA GTA GAA GAG AAG GCT TTC AGC CCA GAA GTA Lys Val Val Glu Glu Lys Ala Phe Ser Pro Glu Val	504

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1	505	ATA CCC ATG TTT TCA GCA TTA TCA GAA GGA GCC ACC Ile Pro Met Phe Ser Ala Leu Ser Glu Gly Ala Thr	540
5	541	CCA CAA GAT TTA AAC ACC ATG CTA AAC ACA GTG GGG Pro Gln Asp Leu Asn Thr Met Leu Asn Thr Val Gly	576
	577	GGA CAT CAA GCA GCC ATG CAA ATG TTA AAA GAG ACC Gly His Gln Ala Ala Met Gln Met Leu Lys Glu Thr	612
	613	ATC AAT GAG GAA GCT GCA GAA TGG GAT AGA GTA CAT Ile Asn Glu Glu Ala Ala Glu Trp Asp Arg Val His	648
10	649	CCA GTG CAT GCA GGG CCT ATT GCA CCA GGC CAG ATG Pro Val His Ala Gly Pro Ile Ala Pro Gly Gln Met	684
	685	AGA GAA CCA AGG GGA AGT GAC ATA GCA GGA ACT ACT Arg Glu Pro Arg Gly Ser Asp Ile Ala Gly Thr Thr	720
15	721	AGT ACC CTT CAG GAA CAA ATA GGA TGG ATG ACA AAT Ser Thr Leu Gln Glu Gln Ile Gly Trp Met Thr Asn	756
	757	AAT CCA CCT ATC CCA GTA GGA GAA ATT TAT AAA AGA Asn Pro Pro Ile Pro Val Gly Glu Ile Tyr Lys Arg	792
	793	TGG ATA ATC CTG GGA TTA AAT AAA ATA GTA AGA ATG Trp Ile Ile Leu Gly Leu Asn Lys Ile Val Arg Met	828
20	829	TAT AGC CCT ACC AGC ATT CTG GAC ATA AGA CAA GGA Tyr Ser Pro Thr Ser Ile Leu Asp Ile Arg Gln Gly	864
	865	CCA AAA GAA CCT TTT AGA GAC TAT GTA GAC CGG TTC Pro Lys Glu Pro Phe Arg Asp Tyr Val Asp Arg Phe	900
25	901	TAT AAA ACT CTA AGA GCC GAG CAA GCT TCA CAG GAG Tyr Lys Thr Leu Arg Ala Glu Gln Ala Ser Gln Glu	936
	937	GTA AAA AAT TGG ATG ACA GAA ACC TTG TTG GTC CAA Val Lys Asn Trp Met Thr Glu Thr Leu Leu Val Gln	972
	973	AAT GCG AAC CCA GAT TGT AAG ACT ATT TTA AAA GCA	1008
30	1009	TTG GGA CCA GCG GCT ACA CTA GAA GAA ATG ATG ACA Leu Gly Pro Ala Ala Thr Leu Glu Glu Met Met Thr	1044

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	1045	GCA TGT CAG GGA GTA GGA GGA CCC GGC CAT AAG GCA Ala Cys Gln Gly Val Gly Gly Pro Gly His Lys Ala	1080
5	1081	AGA GTT TTG GCT GAA GCA ATG AGC CAA GTA ACA AAT Arg Val Leu Ala Glu Ala Met Ser Gln Val Thr Asn	1116
	1117	ACA GCT ACC ATA ATG ATG CAG AGA GGC AAT TTT AGG Thr Ala Thr Ile Met Met Gln Arg Gly Asn Phe Arg	1152
	1153	AAC CAA AGA AAG ATG GTT AAG TGT TTC AAT TGT GGC Asn Gln Arg Lys Met Val Lys Cys Phe Asn Cys Gly	1188
10	1189	AAA GAA GGG CAC ACA GCC AGA AAT TGC AGG GCC CCT Lys Glu Gly His Thr Ala Arg Asn Cys Arg Ala Pro	1224
	1225	AGG AAA AAG GGC TGT TGG AAA TGT GGA AAG GAA GGA Arg Lys Lys Gly Cys Trp Lys Cys Gly Lys Glu Gly	1260
15	1261	CAC CAA ATG AAA GAT TGT ACT GAG AGA CAG GCT AAT His Gln Met Lys Asp Cys Thr Glu Arg Gln Ala Asn	1296
	1297	TTT TTA GGG AAG ATC TGG CCT TCC TAC AAG GGA AGG Phe Leu Gly Lys Ile Trp Pro Ser Tyr Lys Gly Arg	1332
	1333	CCA GGG AAT TTT CTT CAG AGC AGA CCA GAG CCA ACA Pro Gly Asn Phe Leu Gln Ser Arg Pro Glu Pro Thr	1368
20	1369	GCC CCA CCA TTT CTT CAG AGC AGA CCA GAG CCA ACA Ala Pro Pro Phe Leu Gln Ser Arg Pro Glu Pro Thr	1404
	1405	GCC CCA CCA GAA GAG AGC TTC AGG TCT GGG GTA GAG Ala Pro Pro Glu Glu Ser Phe Arg Ser Gly Val Glu	1440
25	1441	ACA ACA ACT CCC CCT CAG AAG CAG GAG CCG ATA GAC Thr Thr Thr Pro Pro Gln Lys Gln Glu Pro Ile Asp	1476
	1477	AAG GAA CTG TAT CCT TTA ACT TCC CTC AGA TCA CTC Lys Glu Leu Tyr Pro Leu Thr Ser Leu Arg Ser Leu	1512
	1513	TTT GGC AAC GAC CCC TCG TCA CAA TAA Phe Gly Asn Asp Pro Ser Ser Gln End	1539

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1 A variety of eukaryotic cells and expression systems
are available for expression of heterologous proteins.
The most widely used among these are yeast, insect and
mammalian systems, although the invention is not limited
5 to use of these. Typically, these systems employ a
recombinant DNA molecule comprising a coding sequence for
the gene of interest operatively linked to a regulatory
element, a selection marker and, in some cases, maintenance
functions such as an origin of replication. A regulatory
10 element is a DNA region or regions which comprise functions
necessary or desirable for transcription and translation.
Typically, the regulatory region comprises a promoter for
RNA polymerase binding and initiation of transcription.

Insect cells which can be used in the invention
15 include Drosophila cells and Lepidoptera cells. Useful
Drosophila cells include S1, S2, S3, KC-0 and D. hydei
cells. See, for example, Schneider et al., J. Embryol.
Exp. Morph. 27:353 (1972); Schulz et al., Proc. Natl.
Acad. Sci. USA 83:9428 (1986); Sinclair et al., Mol. Cell.
20 Biol. 5:3208 (1985). Drosophila cells are transfected by
standard techniques, including calcium phosphate
precipitation, cell fusion, electroporation and viral
transfection. Cells are cultured in accordance with
standard cell culture procedures in a variety of nutrient
25 media, including, e.g., M3 media which consists of
balanced salts and essential amino acids. See, Lindquist,
DIS 58:163 (1982).

Promoters known to be useful in Drosophila include
mammalian cell promoters as well as Drosophila promoters,
30 the latter being preferred. Examples of useful Drosophila
promoters include the Drosophila metallothionein promoter,
the 70 kilodalton heatshock protein promoter (HSP70) and
the COPIA LTR. See, for example, DiNocera et al., Proc.
Natl. Acad. Sci. USA 80:7095 (1983); McGarry et al., Cell
35 42:903 (1985). Conveniently, an expression cassette
comprising the gag coding sequence and regulatory element

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1 can be cloned within a bacterial cloning vector for purposes of propagating the DNA prior to transfection of the animal cells.

In the preferred embodiments of this invention, the 5 HIV gag precursor is expressed in Lepidoptera cells to produce immunogenic gag particles. For expression of the gag precursor protein in Lepidoptera cells, use of a Baculovirus expression system is preferred. In such system, an expression cassette comprising the gag coding 10 sequence and regulatory element is placed into a standard cloning vector for purposes of propagation. The recombinant vector is then co-transfected into Lepidoptera cells with DNA from a wild type Baculovirus. Recombinant viruses resulting from homologous recombination are then 15 selected and plaque purified substantially as described by Summers et al., TAES Bull. NR 1555, May, 1987.

Useful Lepidoptera cells include cells from Trichoplusia ni, Spodoptera frugiperda, Heliothis zea, Autographica californica, Rachiplusia ou, Galleria melonella, Manduca sexta or other cells which can be infected with Baculoviruses, including nuclear polyhedrosis viruses (NPV), single nucleocapsid viruses (SNPV) and multiple nucleocapsid viruses (MNPV). The preferred Baculoviruses are NPV or MNPV Baculoviruses 20 because these contain the polyhedrin gene promoter which is highly expressed in infected cells. Particularly exemplified hereinbelow is the MNPV virus from Autographica californica (AcMNPV). However, other MNPV and NPV viruses can also be employed the silkworm virus, Bombyx mori. 25 Lepidoptera cells are co-transfected with DNA comprising the expression cassette of the invention and with the DNA of an infectious Baculovirus by standard transfection techniques, as discussed above. Cells are cultured in accordance with standard cell culture techniques in a 30 variety of nutrient media, including, for example, TC100 (Gibco Europe; Gardiner et al., J. Inverteb. Pathol. 35

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1 25:363 (1975)) supplemented with 10 % fetal Calf serum
2 (FCS). See, Miller et al., in Setlow et al., eds.,
3 Genetic Engineering: Principles and Methods, Volume 8,
4 New York, Plenum, 1986, pages 277-298.

5 Production in insect cells can also be accomplished by
6 infecting insect larvae. For example, the gag precursor
7 can be produced in Trichoplusia ni caterpillars by feeding
8 the recombinant Baculovirus of the invention along with
9 traces of wild type Baculovirus and then extracting the
10 gag precursor from the hemolymph after about two days.

11 Promoters for use in Lepidoptera cells include
12 promoters from a Baculovirus genome. The promoter of the
13 polyhedrin gene is preferred because the polyhedrin
14 protein is naturally over expressed relative to other
15 Baculovirus proteins. The polyhedrin gene promoter from
16 the AcMNPV virus is preferred. See, Summers et al., TAES
17 Bull. NR 1555, May 1987; Smith et al., EP-A-127,839; Smith
18 et al. Proc. Natl. Acad. Sci. USA 82:8404(1985); and
19 Cochran, EP-A-228,036.

20 For expression in mammalian cells, the expression
21 cassette is likewise cloned within a cloning vector and is
22 then used to transfect the mammalian cells. The vector
23 preferably comprises additional DNA functions for gene
24 amplification, e.g., a DHFR expression cassette, and may
25 also comprise additional functions for selection and/or
26 amplification, e.g., a neomycin resistance cassette for
27 G418 selection. Other functions, such as for transcription
28 enhancement can also be employed. Yet other functions can
29 be comprised within the vector for stable episomal
30 maintenance, if desired, such as maintenance functions of
31 Bovine Papilloma Virus. The mammalian cell vector can
32 also be a recombinant virus, such as a recombinant
33 vaccinia or other pox virus. See, e.g., Paoletti, et al.,
34 U.S. Patent 4,603,112; Paoletti, et al., Proc. Natl. Acad.
35 Sci. U.S. 81:193 (1984).

36 Useful mammalian cells include cells from Chinese

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1 hamster ovary (CHO), NIH3T3, COS-7, CVI, mouse or rat
myeloma, HAK, Vero, HeLa, human diploid cells such as
MRC-5 and WI38, or chicken lymphoma cell lines.
Transfection and cell culture are carried out by standard
5 techniques. Production in mammalian cells can also be
accomplished by expression in transgenic animals. For
example, the gag precursor can be expressed using a casein
promoter and purified from milk.

Promoters useful in mammalian cell lines or mammalian
10 primary cells include the SV 40 early and late gene
promoters, the metallothionein promoter, viral LTR's such
as the Rous sarcoma LTR, the Moloney sarcoma virus (MSV)
LTR or the mouse mammary tumor virus (MMTV) LTR, or the
adenovirus major late promoter and hybrid promoters such
15 as a hybrid BK virus and adenovirus major late promoter.
The regulatory region can also comprise downstream
functions, such as regions for polyadenylation, or other
functions, such as transcription enhancer sequences.

Yeast which can be used in the practice of the
20 invention include those of the genera Hansenula, Pichia,
Kluveromyces, Schizosaccharomyces, Candida and
Saccharomyces. Saccharomyces cerevisiae is the preferred
yeast host. Useful promoters include the copper inducible
(CUP1) promoter, glycolytic gene promoters, e.g., TDH3,
25 PGK and ADH, and the PHO5 and ARG3 promoters. See, e.g.,
Miyanohara et al., Proc. Natl. Acad. Sci. USA 80:1 (1983);
Mellor et al., Gene 24:1 (1983); Hitzeman et al., Science
219:620 (1983); Cabezon et al., Proc. Natl. Acad. Sci. USA
81:6594 (1984).

30 In the case of the gag precursor protein particles
produced in accordance with this invention, it is to be
understood that although particles comprising the gag
precursor are preferred, particles comprising derivatives
of the native gag precursor can also be prepared. For
example, one or more nucleotides or amino acids shown in
35 the sequence above can be deleted, substituted or added

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1 without substantially adversely affecting the immunogenic cross-reactivity with authentic gag epitopes. In other words, such derivatives immunologically similar to authentic gag particles in that they are recognized by
5 antibodies raised against at least one of p17, p24 and p16. Such derivatives, while they may include amino acids from other regions, including antigenic regions of the HIV genome, do not encode other HIV functions, such as the protease function of the pol region or the reverse
10 transcriptase function. In addition, such derivatives retain the ability to form particles in insect cell culture as disclosed herein. In this case, it is within the skill of the art to prepare gagd particles comprising hybrid proteins having one or more epitopes additional to
15 the gag epitopes. Such additional epitopes can be of HIV origin or can be derived from other pathogenic organisms, e.g., Hepatitis B Virus or Herpes Virus.

The gag precursor protein is expressed in secreted form and in membrane bound form. It is isolated from
20 conditioned medium by standard techniques of protein isolation and purification. Detergents can be added in order to free the protein from cell membrane material. Following treatment with detergent, e.g., Triton X100, a Tween or sodium dodecyl sulfate (SDS), the protein or
25 particles can be purified by a series of ultrafiltration steps, ultracentrifugation steps, selective precipitations with, e.g., ammonium sulfate or PEG, density gradient centrifugation in CsCl or sucrose gradients and/or chromatographic steps, such as affinity chromatography, immunoaffinity chromatography, HPLC, reversed phase HPLC, cation and anion exchange, size exclusion chromatography and preparative isoelectric focusing. During or following purification, the protein or particles can be treated with, e.g., formaldehyde, glutaraldehyde or NAE to enhance
30 stability or immunogenicity. In view of the discovery herein disclosed that the gag precursor can form
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1 immunogenic particles in the absence of other viral
functions, it is believed that when gag precursor is
expressed in non-particulate form, it can be caused to
form particles synthetically, as has been shown to be the
5 case for the hepatitis B surface antigen following
expression in yeast. See, e.g., EP-A-135,435. Such gag
precursor protein particles are encompassed within the
scope of this invention.

10 The HIV gag precursor protein and particles produced
in accordance with this invention are useful as diagnostic
agents for detection of exposure to HIV. The protein and
particles are also useful in vaccines for the prevention
of infection or for the inhibition or prevention of
disease progression.

15 The Examples which follow are illustrative but not
limiting of the invention. Restriction enzymes and other
reagents were used substantially in accordance with the
vendors' instructions.

20 Examples

Example 1. Vector Construction

25 pRIT12982 (DT 12-16) is a vector which comprises a
1305 base pair (bp) coding sequence for the N-terminal
region of gag precursor protein. It was prepared by
ligating a ClaI-BglII fragment of the gag precursor
protein coding region derived from an HIV genomic clone
(Shaw et al., Science 226:1165 (1984)) to a synthetic
oligonucleotide having the N-terminal coding sequence of
the gag precursor protein. The oligonucleotide has the
30 sequence:

5' C ATG GGT GCT AGA GCT TCC GTG TTG TCC GGT GGT GAA TTG GAT 3'
CCA CGA TCT CGA AGG CAC AAC AGG CCA CCA CTT AAC CTA GC
NcoI Clal

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pRIT12983 is a vector which comprises a 250 bp region
which codes for the C-terminal portion of gag precursor

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1 protein. It was prepared by ligating a BgIII-MaeIII
5 fragment of the gag precursor coding region derived from
an HIV genomic clone to a synthetic oligonucleotide having
the C-terminal coding sequence of the gag precursor
5 protein. The oligonucleotide has the sequence:

STOP
5' G TCA CAA TAA AGA TAG GAT CC 3'
TT ATT TCT ATC CTA GGA GCT
10 MaeIII XhoI.

The 1305 base pair (bp) BamHI(NcoI)-BgIII fragment
from pRIT12982 was ligated to the 250 bp
BgIII-TAA-BamHI-XhoI fragment from pRIT12983 in pUC 12
15 which had been previously cut with BamHI and SalI. The
resulting plasmid, identified as pRIT13001, therefore
contains the entire coding region for the gag precursor
protein on a BamHI(NcoI)-BamHI cassette.

A baculovirus expression vector was prepared by
20 inserting the BamHI fragment from pRIT13001 into the BamHI
site in pAc373. See, Smith, et al., Proc. Natl. Acad.
Sci. USA 82:8404(1985). pAc373 is a baculovirus transfer
vector containing a modified polyhedrin gene into which a
foreign gene can be cloned into a BamHI site and expressed
25 under the control of the strong polyhedrin promoter. See
Summers, et al., Texas Agricultural Exp. Station Bulletin
NR 1555 (May 1987). A derivative of plasmid pAc373 having
a small deletion present far upstream the strong
polyhedrin promoter was also used as an expression
vector. The slight modification did not appear to affect
30 in vitro expression or growth of the recombinant virus.
Insertion of the gag coding sequence into the Baculovirus
vector resulted in plasmid pRIT13003.

A mammalian cell expression vector was prepared by
35 ligating the BamHI fragment from pRIT13001 downstream of

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1 the SV40 late promoter in pSV529 (Gheysen et al., J. Mol.
5 Appl. Genet. 1:385 (1982)). This vector is identified as
pRIT13002.

10 A yeast expression vector was prepared as follows. An
NcoI-BglII fragment was isolated from pRIT12982 and
inserted into a yeast plasmid downstream of and in-frame
with the ARG3 promoter (see, Cabezon et al., Proc. Natl.
15 Acad. Sci. USA 81:6594 (1984)) giving rise to the vector,
pRIT12984 (DT14-20). The C-terminal portion of the gag
precursor protein was isolated from pRIT12983 as a
BglII-BamHI fragment and was inserted into the BglII site
of pRIT 12984, giving rise to the yeast vector, pRIT12985
(DT16-26). pRIT12985 thus comprises a coding sequence for
the full gag precursor, devoid of other HIV sequences,
15 operatively linked to the ARG3 promoter. In addition, it
comprises replication functions from the yeast 2 micron
vector and a URA3 gene selection marker.

Example 2. Expression in Insect Cells

20 Recombinant Baculovirus transfected with pRIT13003
were prepared substantially as described by Summers, et
al., TAES Bull. NR 1555, May 1987, cited above.

25 Spodoptera frugiperda (S.f.) cells were cotransfected
with wild type (wt) AcMNPV Baculovirus DNA and plasmid
pRIT13003 at 1 µg and 50 µg, respectively. Resulting
virus particles were obtained by collecting the
supernatants. The virus-containing media were used to
infect S.f. cells in a plaque assay. Subsequent infection
of S.f. cells using the viral particles which include both
30 wt AcNPV DNA and DNA recombined with the DNA encoding the
p55 gag precursor protein resulted in cells expressing the
gag protein instead of the polyhedrin protein.

35 The "clear plaques" (0.1 - 0.01% frequency) obtained
in the plaque assay were further screened by filter
hybridization with a gag specific probe. Plaques which
hybridized to the gag probe were scored and subsequently

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1 further plaque purified (2-3 times) before a virus stock
was generated; the virus stock was also tested by ELISA.
S.f. cells were then infected with these recombinant gag
virus stocks at a multiplicity of infection (MOI) of 1-10
5 and after 24 hr, 48 hr, 3 days and 5 days, aliquots of the
conditioned medium (Supernatant) and/or cells were treated
with Triton X100 to a final concentration of 1% and
assayed.

The gag precursor protein synthesized in infected
10 insect cells was observed in Western blots using p55
polyclonal antibodies or antiserum from a pool of AIDS
patients (Zairan). A pre-dominant band at molecular
weight (Mr) of 54 kilodaltons (kd) was observed with all
15 tested sera and with p55 polyclonal antisera. A band at
Mr 54 kd was also detected when testing conditioned medium
after 48 hr, 3 days and 5 days. Bands at Mr 49 kd and Mr
47 kd (minor) and a band at Mr 30 kd could also be seen
when cell extracts were analyzed. This latter band with
apparent Mr 30 Kd is only detected with p55 polyclonal
20 antibodies and not with serum of AIDS infected persons.
It was observed that at least 10 times more p55 "epitopes"
expressed in S.f. cells than in Molt cells infected with
HIV (Molt/HTLV-III) and about 80 times more p55 "epitopes"
25 were present in the conditioned medium of S.f. cells
infected with a gag recombinant virus than in the
conditioned medium of Molt/HTLV-III cells.

In a second assay experiment, ultrafiltration (100,000
x g, 1 hr.) of the 48 hr, 3 day and 5 day conditioned
media (2 ml to 200 ml) resulted in a small pellet which
30 was analysed on SDS-gels and which was also analysed by
immunoblotting. One band at Mr 55 kd was recognized with
specific antibodies against p17, p24 and p55,. Only very
small amounts of degraded products at Mr 49-46 could be
detected. On Coomassie-stained gels, a band at 55 kd
35 could be seen which was 20-80% pure. This band
corresponded with the immunoblot and was recognized by
antibodies against p17, p24 and p55 polypeptides.

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1 In a third assay experiment, centrifugation (1 ml) of
the 48 hr, 3 day and 5 day conditioned media in a
microfuge at 12000 rpm for 5 to 20 minutes produced a band
on SDS-gels at Mr 55 kd which was specific for HIV-I p55
5 gag precursor as revealed by antibodies against p17, p24
and p55 polypeptides and as compared to the HIV cell
lysate (Molt/HTLV-III) 55 kd band.

In a fourth assay experiment, 48 hr, 3 day and 5 day
conditioned media (150 ml to 1 liter, containing 1 μ g/ml
10 of aprotinin which was added at 24 hrs. post-infection and
also at the times of harvest) was treated first by
addition of Tween 20 to 0.01% final concentration. Then,
a solution of polyethylene glycol, Mr 6 kd, (PEG6000) (40
% w/v in 2M NaCl) was added to 10 % or 5 % final
15 concentration. After 4 hours at 4°C or preferentially
overnight at 4°C this precipitate was centrifuged at
5000 rpm for 10 min at 4°C. The PEG pellet was then
taken up in 200 μ l to 1 ml HBS-buffer (Hanks balanced
salt, Flow Laboratories, 18-102-54) containing 0.1 % Tween
20 20 and centrifuged in sucrose gradients (20 % - 60 % in
HBS-buffer, 0.1 % Tween 20 at 4°C containing 10 μ l/ml
aprotinin, Sigma Chemical Co., St. Louis, Missouri) for
about 35 min at 50,000 rpm in a Beckman rotor TLA100
(Beckman Instruments, Fullerton, California) at 4°C, or
25 for about 18 hr at 25,000 rpm on a Beckman SW41 rotor at
4°C. Fractions of 0.2 to 0.5 ml, respectively, from
approximately 40-50% sucrose, were collected, frozen at
-20°C and tested either with a specific antigen capture
Elisa assay such as -24/Ig AIDS antiserum biotinylated or
30 AIDS antiserum/Ig core POD (HIV-1 anticore EIA, Abbott
Laboratories). One OD Elisa pick was detected,
demonstrating that on sucrose gradients the p55 gag
protein migrated as particles or "aggregated structures".
The pick fractions and the surrounding fractions were
35 immunoblotted with p17, p24 or p55 antibodies. One major
band at Mr 55 kd in the SDS-reducing gels was detected

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1 corresponding to p55 gag precursor protein as compared to
an extract of Molt/HILV-III cells prepared substantially
as described above.

5 In a fifth assay experiment, a 5 % PEG6000 precipitate
was prepared substantially as described for the fourth
assay experiment from 150 ml of a S.f. culture which had
been co-infected with the gag precursor recombinant
Baculovirus and with a recombinant Baculovirus which
expressed the HIV envelope protein at a MOI of 3 to 5.
10 The PEG6000 pellet was taken up in 200 ul of HBS-buffer
containing 0.1 % Tween 20. After centrifugation at 15000
x g for 1 min, the supernatant was mixed with 11.5 ml of a
1.5 M CsCl solution (0.3 volumes HBS-buffer, 10 mM
Tris-HCL (pH 8.0), 1 mM ethylenediamine tetraacetate
15 (EDTA), 0.1 % Tween 20 and 10 ug/ml of aprotinin). This
suspension was centrifuged in a Beckman Rotor 50Ti for
about 72 hr at 44000 rpm at 18°. Fractions of 300 ul
each were collected, frozen at -20° and tested with a
specific antigen capture Elisa assay (HIV anticore EIA).
20 Bands at densities of about 1.28 and 1.20 g/cm³ were
recognized, the core-like particle apparently having the
density of 1.28 g/cm³.

25 Electron microscopy confirmed the presence of pre-core
(and core) -like particles in the conditioned medium.
Scanning electron microscopy revealed particles which
apparently were budding onto the cell surfaces.
30 Immunogold transmission electron microscopy revealed
particles which were recognized by p24 and p55
antibodies. Also, p17, p24 and p55 epitopes were
recognized by immunogold labelling after brief treatment
with Triton X100 of purified particles in electron
microscopic preparations. The particles were
approximately spherical and of about 100 - 150 nm in
diameter. The particles display electron luscent centers
35 surrounded by a dark staining ring and an outer shell and
appear to have the majority of the p17, p24, p16 and p55
epitopes on the inside surface of the particle.

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1 This Example, therefore, demonstrates expression and
secretion of HIV pre-core-like (and core-like) particles
comprising immunodeficiency virus gag precursor protein.
The particles comprise predominantly (greater than 90% of
5 total protein) full length gag precursor protein and are
formed in the absence of DNA sequences of viral origin
other than the gag precursor sequence and, hence in the
absence of other viral functions such as the retrovirus
protease and reverse transcriptase.

10 To demonstrate that the HIV gag precursor protein made
in S.f. cells is efficiently myristylated, 3×10^6 cells
in $F25 \text{ cm}^2$ flask, were labelled at 48 hr p.i. with 500
 μCi myristic acid NET-830 (Dupont, Wilmington, Delaware)
for 18 hr after they had been infected with recombinant
15 p55 gag baculoviruses at MOI of 5. Subsequently, the
conditioned medium and the cells were processed separately
for western blotting and SDS-gel radioautography.
Conditioned medium displayed one major band at 55 kd which
was also recognized as gag precursor in western blots as
20 revealed by antibodies against p17, p24, p55. Two other
labelled minor bands were detected at Mr 49-46-47 kd and
were recognized specifically by the same set of antibodies
(p17, p24, p55) in the western blot. Cell lysates made in
1 % triton x 100 and frozen at -20°C displayed on
25 radioautography of the 12.5 % Laemli gel and western blot
respectively band at 55 kd (and a minor band at 58 kd
which apparently corresponded to the translation frame
shift as described for the gag retroviral HIV-1 virus
genome and more prominent bands at Mr 49-47-46 and
30 degradation products at Mr 30-27 kd the latter bands were
not radioactive (containing no myristic acid).

Example 3. Expression in Mammalian Cells:

35 The plasmid pRIT13002 was introduced via the
Ca-phosphate coprecipitation technique (Wigler, et al.,
Cell 16:777(1979)) in CosI and CV1 cells. At 48 hr and

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- 1 110 hr post-transfection, the cells and culture medium were assayed using an ELISA specific for gag antigen expression. Cell extracts (10^6 cells) were adjusted to 1 % Triton X100 or 0.5 % DOC-NP40. The p55 antigen was
- 5 detected using ELISA capture antigen tests involving polyclonal and monoclonal antibodies to p17, p24 or p55 or using the Dupont RIA test (NEK-040), involving a competition with purified p24 peptide. The expression levels obtained were between 4 and 10 ng/ml as measured by
- 10 the p24 RIA Dupont test.

Example 4. Expression in yeast cells

The plasmid pRIT12985 was introduced into the S. cerevisiae strain 02276b (ura3⁻ dur0^h roc1⁻).

- 15 The p55 antigens were detected in yeast extracts (cells in mid-log phase, broken using glass beads or spheroplasting with zymolase). The p55 was detected using ELISA tests, involving polyclonal and monoclonal antibodies to the p24 peptide, or using the Dupont radioimmune assay (RIA)
- 20 involving competition with the purified p24 peptide.

The p55 protein synthesized in S. cerevisiae was observed in Western blots, using p17 or p24 specific monoclonal antibodies, and has a molecular weight similar to that of the p55 antigen obtained from infected cells.

- 25 When cellular extracts were obtained in the absence of detergents, an important fraction of the antigen was retained in the "membrane pellet". This fraction of antigen was recovered using Triton X100. Use of detergents either prior to or after isolation enhanced antigenicity as measured in the RIA. The gag precursor produced in yeast was shown to be myristilated by labelling with tritiated myristic acid and was apparently associated with cell plasma membrane as shown by electron microscopy.
- 30
- 35 The above Examples demonstrate expression of gag precursor protein in animal cell culture and expression of immunodeficiency virus pre-core-like particles in

- 1 Lepidoptera cells using a Baculovirus expression system. The protein and/or particles thus prepared are purified and formulated into a vaccine for parenteral administration to humans in danger of exposure to HIV, in
- 5 order to protect the vaccinees from onset of disease symptoms associated with HIV infection. Each vaccine dose comprises an amount of the protein or particle which is safe, i.e., does not cause significant adverse side effects, but which is effective in inducing an immune
- 10 response. For example, each dose comprises 1 to 1000 ug, preferably 10 to 500 ug, of gag precursor protein or particle in a pharmaceutically acceptable carrier, e.g., an aqueous solution buffered to about pH 5 to 9, preferably pH 6 to 8. The vaccine can also comprise an
- 15 adjuvant, e.g., aluminum hydroxide, muramyl dipeptide or a saponin such as Quil A. Useful buffers include buffers derived from sodium or ammonium cations and acetate, citrate, phosphate or glutamate anions. Other pharmaceutically acceptable carriers or diluents can be
- 20 used to adjust isotonicity or to stabilize the formulation, e.g., sodium chloride, glucose, mannitol, albumin or polyethylene glycol. The vaccine can be lyophilized for convenience of storage and handling. Such vaccine is reconstituted prior to administration.
- 25 Alternatively, the gag protein or particle can be formulated in liposomes or ISCOMS by known techniques. An exemplary vaccine dose comprises 100 ug of gag particles adsorbed on aluminum hydroxide in water buffered to pH 7 with sodium acetate.
- 30 In an alternative embodiment of the invention, the gag protein or particle is mixed with one or more other antigens by coexpression in the same cell culture or by co-formulation. Such other antigens can be other HIV antigens, e.g., antigens derived from the envelope protein, gp160 or gp120, or can be antigens derived from one or more other pathogenic organisms, cells or viruses,
- 35

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1 such as hepatitis B surface antigen for conferring protection against Hepatitis B Virus or antigens derived from the Herpes Virus glycoprotein for conferring protection against Herpes Virus.

5 The vaccine is preferably administered parenterally, e.g., intramuscularly (im) or subcutaneously (sc), although other routes of administration may be useful in eliciting a protective response. The vaccine is administered in a one-dose or multiple-dose, e.g., 2 to 4, 10 course. Immunoprotection can be ascertained by assaying serum anti-gag antibody levels. Thereafter, vaccinees can be revaccinated as needed, e.g., annually.

As a diagnostic reagent, the gag protein or particles can be used in any of the standard diagnostic assays, such 15 as an ELISA or RIA, to detect the presence of anti-HIV antibodies in clinical specimens. Such diagnostic can be used in conjunction with other HIV antigens to monitor disease progression. Use of the gag protein or particle as a diagnostic reagent will generally involve contacting 20 a sample of human or other animal serum or other body fluid with the protein or particle, preferably bound or otherwise affixed or entrapped, and then assaying for binding of anti-gag antibodies from the serum or other sample to the gag protein or particles. Such assay can be 25 accomplished by standard techniques, including by quantitating binding of subsequently added labelled anti-gag antibodies.

Example 5. Construction and Expression of a Mutant p55
30 Gene

In order to examine the potential role of the N-myristoylation in the assembly and formation of extracellular gag particles we have constructed a glycine deletion mutant. Therefore a synthetic oligonucleotide 35 linker syn3 was substituted, for the BamHI-ClaiI fragment in pRIT12982 (see example 1). Syn3 encodes the genuine

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1 N-terminal amino acids of the gag protein except that the second glycine codon is deleted. This mutant BamHI p55 expression cassette was subcloned into the BamHI site of the baculo expression vector pAcYMI (Matsuura et al., J.
5 Gen. Virol. 68:1233 (1987)) and recombinant plaques were obtained and selected essentially as described in Example 1. The recombinant virus, AcGag 31-18, harbouring the glycine deletion mutation of the gag gene was used to infect S.f. cells. The gag precursor protein was
10 efficiently synthesized as determined by an ELISA assay. Metabolic labelling with ³H-myristic acid essentially as described in Example 2 revealed no myristic acid incorporation confirming that deletion of the N-terminal glycine was sufficient to prevent myristylation of the
15 gag precursor protein. Analysis of the cell extracts in Western blots (see Example 1) showed a prominent band of 55 kd and lower M.W. degradation products. The obtained pattern of protein bands was similar to the wild type (wt) p55 protein expressed in S.f. cells. In contrast with the
20 wt p55 recombinant, no gag protein could be detected with the glycine mutant 2 days p.i. using PEG or ultracentrifugation of the conditioned medium. Thus the mutated p55 protein was only detected within the infected cells. The myristylation process thus seems to be
25 required for the extracellular release of the p55 product. Scanning electron microscopy (SEM) revealed that the cell surface was rather smooth, showing no particles. Thin section transmission electron microscopy and immunogold labelling performed on cells infected 24 hrs, 48 and 66
30 hrs p.i. with the Ac gag 31-18 (Myr⁻) recombinant virus revealed that the non-myristylated gag protein was efficiently expressed, scattered in the cytoplasm or associated to grey amorphous structures within the cytoplasm and the nucleus. These intracellular particles
35 or particulate structures are morphologically different

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1 from the extracellular particles obtained with the
myristoylated gag recombinant (AcGag7) as they display a
double electron dense ring structure and do not contain a
lipid bilayer derived from the cell membrane. Neither gag
5 protein nor budding structures were observed at the cell
membrane.

These results demonstrate that the myristylation of
the gag precursor appears to be required for its plasma
membrane location, budding and extracellular particle
10 release. Myristylation does not seem however to be
required for the multimeric assembly of the p55 molecules.
Accumulation of the non-myristoylated p55 products within
the nucleus (and nucleoli) is a surprising phenomenon.

15 Example 6. Construction and Expression of a Truncated p55
Precursor Protein

In order to examine the role of the p16 (COOH-end) of
the HIV precursor gag protein we made a gag deletion
mutant which encodes only the p17-p24 precursor part of
20 gag.

The BamHI-CfrI gag fragment of pRIT13003 was purified
and ligated with a synthetic oligonucleotide sequence
5' GGC CAT AAG GCA AGA GTT TTA GTT AGT TAG 3'
3' TA TTC CGT TCT CAA AAT CAA TCA ATC CTA G 5'
25 and gel purified and cloned in the BamHI-site of pAcYMI.
This linker sequence contains the genuine amino acid
COOH-end of the HIV p24 core protein and two additional
amino acids, Valine and Serine. This recombinant plasmid
was used to co-transfect S.f. cells with AcMNPV DNA
30 essentially as described before (see Example 1).
Recombinant plaques were screened as described in Example
1.

A selected recombinant virus, Ac CfrI, was used to
infect S.f. cells. A truncated gag-polypeptide (p17-24)
35 was detected at the expected M.W. of 41 Kd and which

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1 reacted in Western blot analysis with p17 and p24 monoclonals. The p17-24 product was predominantly expressed inside the cells but a small amount of extracellular p17-24 product could be detected when
5 analyzing the conditioned medium by Western blotting. PEG precipitation and ultracentrifugation of the conditioned medium of the CfrI mutant gag protein did not result in detectable p17-p24 product. Electron microscopy analysis showed no evidence of budding or extracellular gag
10 particles. Large protusions 1-4 μ m long in the form of tubular structures which are longitudinally connected to the cell membrane surface could be detected early in infection. Immunogold labelling showed that the truncated gag protein (p17-p24) was localized at the cell membrane
15 and at the periphery of these tubular extensions, but no electron dense "ring" structures - typical of the p55 particle structures - could be detected. This probably indicates that the p17-p24 product is not able to assemble in multimeric structures, i.e., cap formations, at the
20 cell membrane. These results suggest that at least a part of the p16 polypeptide of the gag precursor polypeptide is necessary for particle formation.

A glycine deletion mutant of the CfrI cassette (non-myristoylated p17-24) was made by exchanging the
25 EcoRV-PstI fragment of 669 bp of the pAcGag 31-18 non-myristoylated p55 gene with the pAC CfrI EcoRV-PstI \pm 9400 bp long fragment. This mutant displayed no protrusions of membranes as described above but showed p17 and p24 immunogold decoration scattered in the cytoplasm
30 and nucleus.

Example 7. Construction and Expression of a gag-pol Protein and the Processed Polypeptides

To express the gag-pol products, we have included most
35 (about 80 %) of the pol gene into the baculovirus transfer vector carrying the p55 expression cassette

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1 (pRIT13003). The pol gene DNA fragment is a BgIII (2093) - EcoRI (4681) restriction fragment from BH10 (Shaw et al., Science 226:1165 (1984). A poly-stop synthetic DNA fragment 5' AAT TCC TAA CTA ACT AAG 3'

5 3'GGA TTG AT TGA TTC CTA G 5'

was added at the Eco RI site. The resulting baculovirus expression plasmid, LE-8-4, was used in a co-transfection experiment to generate recombinant plaques essentially as described in Example 1.

10 In this recombinant construct, the myristoylated p55 as well as a gag-pol product resulting from the HIV-specific translational frame-shift in S.f. cells, are expected to be produced, and subsequently processed by the protease. Recombinant baculovirus harbouring the gag-pol 15 gene was screened and selected essentially as described in Example 1. In S.f. cells infected with such a gag-pol recombinant virus, VAC 8-5, no gag or gag-pol products were detected when the conditioned medium was analysed by Western Blot or precipitated with PEG.

20 Cell extracts however, did show a strong doublet band at 24 Kd and a band at 17 Kd which reacted with p24 and p17 monoclonal antibodies in Western blots. Very small amounts of the precursor p55 band and intermediate 41 Kd (46 Kd) bands could also be detected in Western blots.

25 This indicates that the protease is active in the gag-pol fusion protein, expressed by translational frame-shift in S.f. cells. This results in p17, p24 polypeptides and intermediates (41 Kd, 46-49 Kd, 55 Kd). The large precursor gag-pol product was not detected with our p55, 30 p17 or p24 antibodies.

Electron microscopy showed on rare occasions a few particles budding at the cell membrane. These particles seem to be morphologically similar to the above described p55 particles. Co-infection experiments with recombinant 35 viruses harbouring the p55 and the gag-pol gene did not result in detectable particles

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1 displaying a morphological difference such as a more
condensed (cone-shaped) core (p24) structure, typical of a
mature retroviral (HIV) particle.

5 Example 8. Construction and Expression of the SIV
Pr57^{gag} Gene in S.F. Cells

The gag gene of Simian Immunodeficiency virus (SIV)
was subcloned from the molecularly cloned SIV_{mac}-BK28
(gift of J. Mullins; see Hirsh et al., Cell 49:307 (1987)
10 and Kestler et al., Nature 331:619 (1988)). A 3504 bp KpnI
fragment of the pBK28 genome (nucleotides 1212 to 4716)
was subcloned into pUC8. Two internal fragments of the gag
gene, the 5' fragment FnuDII(1201) - Pst (1959) and the
3' fragment PstI (1959) - HphI (2803) were purified and
15 synthetic oligonucleotide linkers, linker 1:

GAT CC ACC ATG GGC
G TGG TAC CCG

and linker 3:

TGCTGCACCTCAATTCTCTCTGGAGGAGACCACTAGAGATCTGGTAC
20 AACGACGTGGAGTTAAGAGAGAACCTCCTCTGGTCATCTCTAGAC
were ligated to adequate gag fragments to reconstitute the
entire precursor gag gene. In a separate experiment a
linker 2:

GATCC ACC ATG GCC
25 G TGG TAC CCG

was used at the 5' fragment, to introduce a mutation in
the second codon, namely, GGC (Gly) to GCC (Ala). The
different constructions were cloned into blue scribe
vectors and verified by sequencing. The N-terminal
30 fragment (BamHI-PstI) and the carboxy-terminal fragment
(PstI-BglII) were isolated and cloned into the BamHI
digested, alkaline phosphatase treated pAcYMI baculovirus
expression vector. The pAC gag Myr⁺ plasmid contains the
native SIV gag gene and the pAC gag Myr⁻ contains the
35 mutated (Gly to Ala) gene. S.f. cells were transfected
with a mixture of purified AC MNPV viral DNA (1 µg) and

-30-

1 the respective recombinant transfer plasmids (50 µg) essentially as described in Example 1. The recombinant plaques were screened and selected as described in Example 1.

5 The SIV gag Pr57^{gag} precursor polypeptide was efficiently synthesized in infected insect cells as observed in Western blots using the rabbit antiserum to SIV (metrizamide - gradient purified SIV-BK28 virus) or a monoclonal directed against the COOH-end of the HIV p24 10 polypeptide, which appear also to recognize the SIV core protein.

In a second assay experiment it was demonstrated that the SIV native gag precursor gene expressed in S.f. cells was efficiently myristoylated in contrast to the culture 15 infected with the glycine to alanine mutant in which no myristylation of the precursor Pr57^{gag} protein could be detected when analysed on SDS-PAGE and radioautography.

As in the case of HIV-gag precursor protein we also observed gag particle formation and release of particles 20 in the conditioned medium when the infected cultures were analysed by ultracentrifugation, sucrose gradients and electron microscopy (TEM and SEM). Similar SIV-gag Pr57^{gag} particles as those obtained when expressing HIV-p55 precursor gene in S.f. cells were observed. The 25 extracellular gag particles form crescent structures at the cell membrane which assemble into typical buds that closely resemble immature virus budding particles. The SIV Pr57^{gag} as the HIV p55 particles were about 100-120 nm in diameter and showed a light grey translucent center 30 surrounded by a tick dark electron dense ring and an outer lipid bilayer. Experiments with the SIV non-myristoylated (Gly to Ala) mutant confirmed the observations made with the HIV-non myristoylated p55 mutant that N-myristylation is essential for budding and extracellular particle 35 formation.

The difference between the gag precursor protein of HIV and SIV is that the latter forms also intracellular

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1 particles and particulate structure when the native SIV
gag protein (myristoylated) is expressed. This could be
explained as follows : the expression level is about 3
times higher than the HIV gag expression level and maybe
5 not all the SIV Pr57^{gag} molecules are myristoylated.
Also more degradation products, especially a myristoylated
p27 protein band could be detected in WB of cultures
infected with the SIV Pr57^{gag} native construct.

It is possible that the cellular structures of about
10 40 nm in diameter and sometimes up to 1 μm long - which
are observed at late stage of infections - are composed at
least in part of these degradation gag products. This
could resemble the p24 core assembly into tubular
structures observed in some rare cases of retroviral core
15 maturation. Also when the p24 core protein of HIV-1 is
expressed in *E. coli*, tubular structures containing p24
protein have been observed. Part of the intracellular
particles observed with the native SIV gag precursor
recombinant take form near the cell membrane, where they
20 appear to differentiate into virus-like particles budding
as described above. This process is reminiscent of the
viral maturation of type D retrovirus which involves
intermediate intracellular type A particles.

The above description and examples fully disclose the
25 invention and the preferred embodiments thereof. The
invention, however, is not limited to the embodiments
specifically disclosed herein but, rather, encompasses all
improvements, variations and modifications thereof which
come within the scope of the following claims.

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CLAIMS:

1. A recombinant DNA molecule comprising a DNA sequence which codes for a full length immunodeficiency virus gag precursor protein and which is devoid of the naturally occurring 5' and 3' flanking sequences, operatively linked to a regulatory element which functions in eukaryotic cells.
- 5 10 2. The recombinant DNA molecule of claim 1 in which the regulatory element is one which functions in yeast, insect or mammalian cells and the gag precursor protein is the HIV gag precursor protein.
- 15 3. The recombinant DNA molecule of claim 2 in which the regulatory element is one which functions in Lepidoptera cells.
4. The recombinant DNA molecule of claim 3 in which the regulatory element comprises the polyhedrin gene promoter.
- 20 5. A recombinant Baculovirus comprising the recombinant DNA molecule of claim 2, 3 or 4.
6. An insect cell infected with the recombinant Baculovirus of claim 5.
7. The insect cell of claim 6 which is a Lepidoptera cell.
- 25 8. The insect cell of claim 6 which is a Spodoptera frugiperda cell.
9. The recombinant DNA molecule of claim 2 in which the regulatory element is one which functions in 30 Drosophila cells.
10. A Drosophila cell transformed with the recombinant DNA molecule of claim 9.
11. The recombinant DNA molecule of claim 2 in which the regulatory region is one which functions in mammalian cells.
- 35 12. A recombinant vaccinia virus comprising the recombinant DNA molecule of claim 11.

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- 1 13. A mammalian cell comprising the recombinant DNA molecule of claim 11.
14. A mammalian cell infected with the recombinant vaccinia virus of claim 12.
- 5 15. The mammalian cell of claim 13 which is selected from the group consisting of CHO cells, COS-7 cells, NIH-3T3 cells, CV1 cells, mouse or rat myeloma cells, HAK cells, vero cells, HeLa cells, WI38 cells, MRC-5 cells or chicken lymphoma cells.
- 10 16. The recombinant DNA molecule of claim 2 in which the regulatory region is one which functions in yeast.
17. The recombinant DNA molecule of claim 16 in which the regulatory element comprises the CUP1, TDH3, PGK, ADH, PHO5 or ARG3 promoter.
- 15 18. A recombinant yeast cell comprising the recombinant DNA molecule of claim 16.
19. A recombinant *S. cerevisiae* cell comprising the recombinant DNA molecule of claim 17.
- 20 20. A recombinant DNA molecule for expressing in Lepidoptera cells a particle which is immunologically similar to authentic immunodeficiency virus gag particles which molecule comprises a DNA sequence which codes for all or a portion of an immunodeficiency virus gag precursor protein or for a hybrid protein having all or a portion of an immunodeficiency virus gag precursor protein, operatively linked to a regulatory element which functions in Lepidoptera cells.
- 25 21. The recombinant DNA molecule of claim 20 for expressing a particle comprising predominantly full length HIV gag precursor protein which codes for full length HIV gag precursor protein devoid of other HIV functions.
- 30 22. A recombinant DNA molecule comprising a coding sequence for an immunodeficiency virus gag precursor protein operatively linked to a regulatory region which functions in Lepidoptera cells.
- 35 23. The recombinant DNA molecule of claim 22 in which the coding sequence is for a full length HIV gag precursor protein devoid of other HIV functions.

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- 1 24. The recombinant DNA molecule of claim 20, 21, 22 or 23 in which the regulatory element comprises the polyhedrin gene promoter.
- 5 25. A recombinant Baculovirus comprising the recombinant DNA molecule of claim 20 or 22.
26. A recombinant Baculovirus comprising the recombinant DNA molecule of claim 24.
27. A Lepidoptera cell infected with the recombinant Baculovirus of claim 25.
- 10 28. A Spodoptera frugiperda cell infected with the recombinant Baculovirus of claim 26.
29. A gag precursor protein produced by culturing cells of claim 6.
- 15 30. A gag precursor protein produced by culturing cells of claim 13 or 18.
31. A gag precursor protein produced by culturing cells of claim 27.
32. A gag precursor protein particle isolated from conditioned medium from a culture of cells of claim 6.
- 20 33. A gag precursor protein particle produced by culturing cells of claim 27.
34. An immunogenic particle comprising gag precursor protein produced by recombinant eukaryotic cells which particle is immunologically similar to authentic immunodeficiency virus 25 gag particles.
35. The immunogenic particle of claim 34 which comprises predominantly full length HIV gag precursor protein, which is recognized by anti-p16, anti-p24 and anti-p17 antibodies and which lacks viral functions required for viral maturation and 30 replication.
36. A vaccine comprising gag precursor protein produced by recombinant eukaryotic cells.
37. A vaccine comprising gag precursor protein particles produced by recombinant eukaryotic cells.
- 35 38. A method for collecting data useful in the diagnosis of exposure of an animal to an immunodeficiency virus which

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1 comprises contacting a sample of serum or other bodily fluid from the animal with a gag precursor protein of claims 29, 30, 31 or 32.

5 39. A method for collecting data useful in the diagnosis of exposure of an animal to an immunodeficiency virus which comprises contacting a sample of serum or other bodily fluid from the animal with the immunogenic particle of claim 34 or 35.

40. The gag precursor protein of any of claims 29, 30, 31 or 32 for use as a vaccine agent.

10 41. The gag precursor protein of any of claims 29, 30, 31 or 32 for use as a vaccine agent for conferring protection in humans against infection by HIV.

15 42. The gag precursor protein of any of claims 29, 30, 31 or 32 for use in the manufacture of a vaccine for conferring protection in humans against infection by HIV.

43. The immunogenic particle of claim 34 or 35 for use as a vaccine agent.

44. The immunogenic particle of claim 34 or 35 for use as a vaccine agent for conferring protection in humans against 20 infection by HIV.

45. The immunogenic particle of claim 34 or 35 for use in the manufacture of a vaccine for conferring protection in humans against infection by HIV.

46. A process for preparing a recombinant DNA molecule 25 for expressing an immunodeficiency virus gag protein which comprises ligating a DNA sequence which codes for a full length immunodeficiency virus gag precursor protein and which is devoid of the naturally occurring 5' and 3' flanking sequences to a regulatory element which functions in eukaryotic cells.

30 47. The process of claim 46 in which the regulatory element is one which functions in yeast, insect or mammalian cells and the gag precursor protein is the HIV gag precursor protein.

48. The process of claim 47 in which the regulatory 35 element is one which functions in Lepidoptera cells.

49. The process of claim 48 in which the regulatory element comprises the polyhedrin gene promoter.

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1 50. A method of preparing a vaccine for protecting a
human against disease caused by infection by HIV which
comprises culturing recombinant eukaryotic cells which have
been transformed with a recombinant DNA molecule comprising a
5 DNA sequence which codes for a full length HIV gag precursor
protein and which is devoid of naturally occurring 5' and 3'
flanking sequences, operatively linked to a regulatory element
which functions in the eukaryotic cells; isolating the gag
precursor protein particles produced thereby; and combining the
10 isolated gag precursor protein particles with a pharmaceutically
acceptable carrier.

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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/02415

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4) : C 12 N 5/00, 15/00; C 07 H 15/12; A 61 K 39/00

II. FIELDS SEARCHED

Classification System	Minimum Documentation Searched ?
	Classification Symbols
U.S.	435/240.2, 255, 320; 424/88; 536/27
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *	

Dialog Biotech File, 1967-1989; CAS File 1967-1989

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages ***	Relevant to Claim No. ****
Y	Virology, Volume 158, published March 1987	1-10, 46-50
X	L. Madisen, et al. "Expression of the Human Immunodeficiency Virus gag gene in insect cells" pp. 248-250. see entire article.	20-28
Y	US, A, 4,703,008 (Lin) 27 October 1987. see entire document.	11-15
Y	WO, A, 87/02038 (Oncogen) 09 April 1987. see entire document.	11-15, 50
Y	Science, Volume 231, published 28 March 1986 R.A. Kramer, et al. "HTLV-III gag protein is processed in yeast by the virus pol-protease" pp. 1580-1584. see entire article.	16-19
Y	FEBS Letters, Volume 204, Number 1-published August 1986. M. Hoylearts, et al. "High-level production and isolation of human recombinant alpha 1 proteinase inhibitor in yeast" pp. 83-87, see entire article.	17

* Special categories of cited documents: **

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

09 December 1989

Date of Mailing of this International Search Report

20 DEC 1989

International Searching Authority

Signature of Authorized Officer

ISA/US

Beth A. Burrous

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Mol. and Cell. Biol., Volume 6, Number 4, published April 1986. D. Thiele, et al. "Tandemly duplicated upstream control sequences mediate copper-induced transcription of the <u>Saccharomyces cerevisiae</u> copper-metallothionein gene" pp. 1158-1163. see entire article.	17
Y	Proc. Natl. Acad. Sci. USA., Volume 81, published November 1984. T. Cabezon, et al. "Expression of human alpha 1-antitrypsin c DNA in the yeast <u>saccharomyces cerevisiae</u> " pp. 6594-6598. see entire article.	17
Y	Gene, Volume 33, published January 1985. J. Mellor, et al. "Factors affecting heterologous gene expression in <u>saccharomyces cerevisiae</u> " pp. 215-226, see entire article.	17
Y, P	US, A, 4,778,761 (Myanohara) 18 October 1988 see entire document.	17